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EVIDENCE FOR ECDYSTEROID FEEDBACK ON RELEASE OF MOLT-INHIBITING HORMONE FROM CRAB EYESTALK GANGLIA

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Abstract

The content and release of molt-inhibiting hormone activity (MIH) in isolated eyestalk ganglia of crabs (*Cancer antennarius* S.) were measured *in vitro* as a function of exposure *in vivo* to elevated or reduced hemolymph ecdysteroid levels. Ecdysteroid titers of intermolt crabs injected with 20-hydroxyecdysone (two 45 μg injections/24 h) rose 8- to 10-fold; MIH released from subsequently isolated ganglia was significantly less than that released from ganglia of saline-injected controls, while MIH content of ganglia from treated crabs was increased. The hemolymph ecdysteroid level of intermolt crabs was low (6 ng/ml) and was further reduced by 40% 6 days after Y-organectomy. MIH release from ganglia of both Y-organectomized and sham-operated control crabs was high, similar to that of unoperated controls, but MIH content of ganglia from both Y-organectomized and sham groups was significantly reduced relative to controls. The results indicate a negative feedback regulation of MIH release but not synthesis by ecdysteroids and are discussed in relation to the patterns of ecdysteroid titers observed in the normal crustacean molt cycle.

Introduction

In vertebrates, steroids produced by neuropeptide-regulated glands exert feedback actions on production and/or release of the tropic neurohormones. Feedback effects are generally negative as in adrenal glucocorticoid inhibition of corticotropin-releasing factor and adrenocorticotropin (ACTH) release from the hypothalamus and the pituitary, respectively (see Keller-Wood and Dallman, 1984 for review); feedback of gonadal steroids on gonadotropin release is predominantly negative although positive effects are seen under certain physiological circumstances (Baldwin *et al.*, 1974; Martin *et al.*, 1974; Labrie *et al.*, 1978). Insect neurosecretion of brain prothoracicotropic hormone (PTTH) is apparently subject to inhibition by the ecdysteroid molting hormone, ecdysone, which is produced by the target prothoracic glands (Steel, 1975). In these cases neuropeptide regulation of target cell steroidogenesis is positive, and negative feedback provides a homeostatic mechanism for maintaining circulating steroid hormone levels within a relatively narrow range. The ecdysteroidogenic glands of crustaceans (Y-organs), on the other hand, are subject to negative regulation by the eyestalk neuropeptide molt-inhibiting hormone (MIH; *cf.* Skinner, 1985); the existence of a corresponding feedback arrangement is probable but has not been explored.

The crustacean molt-controlling system consists of eyestalk neurosecretory cells (X-organs, XO) with enlarged axonal endings contained in the neurohemal sinus gland

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Abbreviations: GCS, eyestalk ganglia-conditioned saline; MIH, molt-inhibiting hormone; PTTH, prothoracicotropic hormone; SG, sinus gland; XO, X-organ.

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(SG) and paired peripheral steroidogenic Y-organs which produce ecdysone (Chang *et al.,* 1976; Cooke and Sullivan, 1982; Watson and Spaziani, 1985a). MIH is a heat-stable, trypsin-sensitive peptide (Rao, 1965; Quackenbush and Herrnkind, 1983) which by immunological and functional criteria appears to be related to neurohypophysial peptides of the vasopressin family (Mattson and Spaziani, 1985a). Serotonergic neurons mediate release of MIH (Mattson and Spaziani, 1985b, 1986a) and one of the conditions governing the release of MIH is stress (Mattson and Spaziani, 1985c, 1986a). Recent advances in Y-organ culture methods have allowed demonstration of a direct suppression of Y-organ ecdysteroid production by MIH activity in eyestalk extracts (Watson and Spaziani, 1985b; Mattson and Spaziani, 1985d, Webster, 1986), sinus gland-conditioned saline (Soumoff and O'Connor, 1982; Mattson and Spaziani, 1985b, Webster, 1986), and eyestalk ganglia-conditioned saline (GCS; Mattson and Spaziani, 1985b). *In vitro* methods also show that cyclic AMP mediates this inhibition of steroidogenesis (Mattson and Spaziani, 1985a, e) and that calcium-calmodulin antagonizes the MIH effect by activating Y-organ cAMP phosphodiesterase (Mattson and Spaziani, 1986b). Thus the effects of cAMP on Y-organ steroidogenesis are opposite to those of the tropic hormones on steroidogenic glands of both vertebrates (Schimmer, 1980; Sala *et al.,* 1979) and insects (Smith *et al.,* 1984).

Recent development of a formal MIH bioassay based upon *in vitro* suppression of Y-organ ecdysteroid production (Mattson and Spaziani, 1985d) allowed assessment of neurotransmitter regulation of release of MIH activity from isolated crab eyestalk ganglia (Mattson and Spaziani, 1985b). The present study employs the MIH bioassay and isolated ganglia techniques to assess possible feedback regulation of MIH release by ecdysteroids. Circulating ecdysteroid titers were artificially elevated or suppressed by injection of 20-hydroxyecdysone or Y-organectomy, respectively; eyestalk ganglia were subsequently isolated and ganglionic content and release of MIH was quantified by bioassay.

Materials and Methods

Animals and experimental treatment

Female rock crabs *Cancer anternnarius* Stimpson (Marinus Inc., Westchester, California; Pacific Biomarine, Venice, California) were used in all experiments; animals were in intermolt upon sacrifice at the end of experiments (staged by examination of the continuity of endodermis and carapace; *cf.,* Skinner, 1985). Crabs were maintained individually in water table compartments containing constantly-recirculating, charcoal-filtered, reconstituted seawater at 16–17°C. A 12/12 h light/dark photocycle was maintained; crabs were fed fish three times weekly and were allowed to acclimate to their environment for at least one week prior to experimentation to reduce possible stress effects on the XO–Y-organ axis (Mattson and Spaziani, 1985c). Hemolymph samples were withdrawn in 300 μl volumes from the sinus at the base of the fourth walking leg, while treatments were administered by injection (300 μl volumes) through the periarthrodal membrane at the base of the first walking leg. Twenty (20)-hydroxyecdysone was obtained from Sigma Chemical Co. (St. Louis, Missouri) and injected at a dose of 45 μg/300 μl; this concentration (10^{-3} *M*) was estimated to give an initial hemolymph concentration of 10^{-5} *M* based upon an average hemolymph volume/crab of 30 ml. Y-organ ablation was carried out on animals chilled for 1 h at 4°C. A 3–5 mm diameter hole was made through the ventral carapace using a portable dental drill with a rough burr bit. Y-organs were ablated by cautery and the carapace opening sealed with paraffin wax. For sham operations, holes were drilled 1 cm distolateral to

the Y-organ site and underlying tissue was cauterized. Crabs were examined at sacrifice with the aid of a dissecting microscope to verify Y-organ destruction.

Eyestalk ganglia incubation and extract preparation

At the end of treatment periods eyestalks were extirpated, placed in Pantin's saline (Pantin, 1934), and the entire optic ganglia complexes (including the intact X-organ-sinus gland system) were removed to saline as reported previously (Mattson and Spaziani, 1985b). Ganglia were incubated (1 ganglion complex/100 μl saline) for 2 h at 20°C in an atmosphere of 50% O_2/50% room air with constant rotary shaking at 60 rpm; we previously found that 2-h incubations resulted in release of MIH activity which was intermediate to shorter (30-min) or longer (8-h) incubations and thus allowed for greater sensitivity in detection of changes in release in response to experimental treatments (Mattson and Spaziani, 1985b). After incubations, the ganglia-conditioned saline (GCS) was removed, placed in a boiling water bath for 2 min, and centrifuged at 1000 × *g* for 10 min. The supernatant volume was adjusted to the preincubation volume with glass distilled water and stored frozen for MIH bioassay. Ganglia extracts were prepared by homogenization in saline (2 ganglia/100 μl) followed by heat treatment in a boiling water bath as previously described (Mattson and Spaziani, 1985d). A dose of two eyestalk equivalents of extract was used for bioassay of MIH; this dose was previously determined to be near the ED_{50} for inhibition of Y-organ steroidogenesis (Mattson and Spaziani, 1985d) and thus allowed for maximal sensitivity of the bioassay to changes in MIH content of ganglia due to experimental treatments.

Bioassay of MIH activity

The MIH bioassay has been previously described (Mattson and Spaziani, 1985b, d). Briefly, activated Y-organs from 48-h de-eyestalked crabs were removed, quartered, and placed in 0.5 ml of fetal bovine serum-supplemented Medium 199. To the incubation medium was then added either 100 μl of saline (for determination of basal ecdysteroid production), eyestalk extract, (2 eyestalk equivalents) or GCS (1 eyestalk equivalent). Incubations were for 24 h, after which incubation medium was removed and stored at 4°C for ecdysteroid RIA, while tissue was processed for protein quantification. The relative ability of GCS or eyestalk extracts (see figure legends) to inhibit Y-organ steroidogenesis was used as a measure of MIH activity (Mattson and Spaziani, 1985b, d). The ecdysteroid contents of GCS and ganglia extracts (at concentrations used for the MIH bioassay) *per se* were below the limit of detection of the ecdysteroid RIA.

Ecdysteroid and protein quantification

Serum and incubation medium were assayed directly for ecdysteroids by RIA as previously described (Mattson and Spaziani, 1985c, d). The RIA utilized ^{3}H-ecdysone (60 Ci/mmol; New England Nuclear, Bedford, MA), ecdysone antiserum (antibody H-21B; Horn *et al.*, 1976) which was a gift from Dr. W. E. Bollenbacher (Dept. of Biology, University of North Carolina, Chapel Hill, NC), and ecdysone standards (Research Plus, Bayonne, NJ). Inter- and intra-assay coefficients of variation were 8% and 4%, respectively. Ecdysone antiserum H-21B has a 10-fold greater affinity for ecdysone than 20-hydroxyecdysone (Horn *et al.*, 1976; Watson and Spaziani, 1985b); as crab hemolymph contains predominantly 20-hydroxyecdysone (Chang *et al.*, 1976) actual ecdysteroid titers for hemolymph in this study are likely an order of magnitude higher than the values presented. The Y-organs of *C. antennarius in vitro* secrete, in

addition to ecdysone, an unknown, less polar, ecdysteroid (structural analysis is in progress); this unknown appears in quantities 5-fold greater than ecdysone but has an affinity for antiserum H-21B 100-fold less than ecdysone (Watson and Spaziani, 1985b). Thus, ecdysone levels that we report in the medium of MIH bioassay runs are underrepresented by well under an order of magnitude. Protein was quantified by the Bradford (1976) method. Statistics were done by Students *t*-test and all values are expressed as mean and standard error of the mean (SEM).

Results

Serum ecdysteroid titers of crabs given injections of 45 μg of 20-hydroxyecdysone at 0-h and 18 h later were elevated 3- and 8-fold 18- and 24-h, respectively, after the initial injection (Fig. 1). Y-organectomy reduced hemolymph ecdysteroid levels over a 6-day period to 65% of levels in sham-operated controls (Fig. 2). Eyestalk ganglia removed from crabs with elevated ecdysteroid levels (24 h after initial 20-hydroxyecdysone injection; *cf.* Fig. 1) released significantly less MIH activity during a 2-h incubation in saline than did control ganglia; ganglia-conditioned saline from controls inhibited basal Y-organ steroidogenesis by 42%, while that from crabs with elevated ecdysteroid titers did not affect ecdysteroid production significantly (Fig. 3). Isolated ganglia from Y-organectomized animals with reduced ecdysteroid titers (*cf.*, Fig. 2) released MIH activity at levels similar to ganglia from sham-operated controls or from unoperated controls (Y-organ steroidogenesis was suppressed 40–50%; Fig. 3). Extracts of eyestalk ganglia from the ecdysteroid-treated crabs contained significantly more MIH activity than ganglia extracts from control crabs (Fig. 4); Y-organ steroidogenesis was inhibited 65% and 80% by extracts from control and 20-hydroxyecdysone-injected crabs, respectively. Figure 4 also shows that MIH activity contained in extracts of ganglia from Y-organectomized crabs and from sham operates was significantly less than that in ganglia from unoperated controls (40% suppression of Y-organ ecdyste-

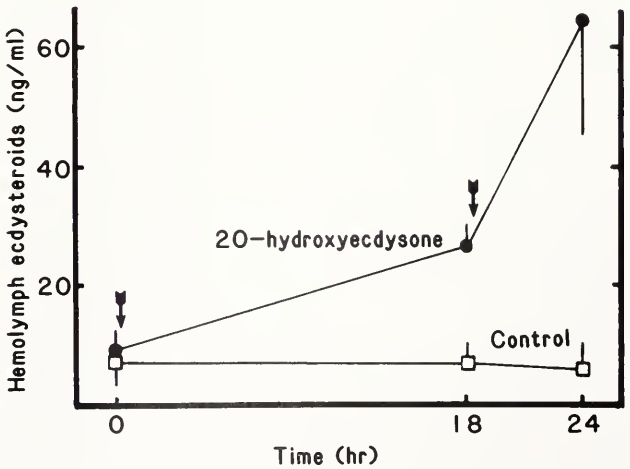

FIGURE 1. Hemolymph ecdysteroid titers after 20-hydroxyecdysone administration. Crabs were injected with saline (control) or 45 μg of 20-hydroxyecdysone (arrows) after serum sampling at the given times. Ecdysteroids were quantified by RIA. Points and lines are the mean and SEM of samples from 4 crabs from 1 of 2 duplicate experiments. Values for 20-hydroxyecdysone-injected crabs > control at 18- and 24-h ($P < 0.01$); 24-h 20-hydroxyecdysone value > 18-h 20-hydroxyecdysone value ($P < 0.05$).

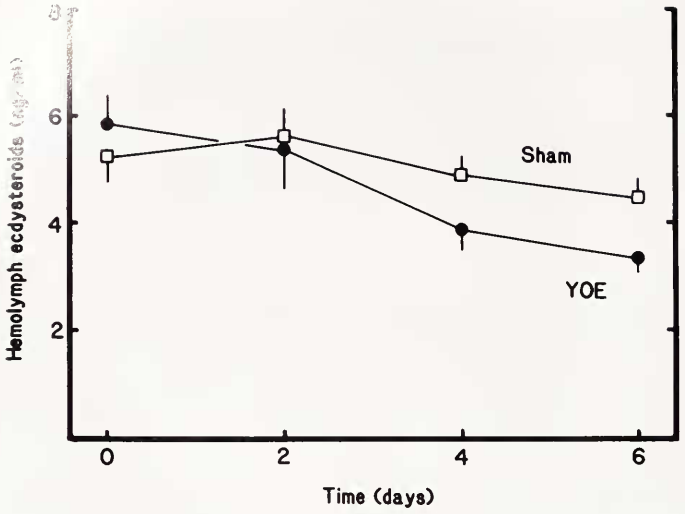

FIGURE 2. Effects of Y-organ removal on hemolymph ecdysteroid levels. Y-organectomy or sham operations were performed after serum sampling at time 0; hemolymph ecdysteroid levels were monitored at the given times thereafter. Points and lines are the mean and SEM from 4 to 5 crabs. Values for Y-organectomized crabs < corresponding sham values at days 4–6 ($P < 0.05$); combined values for sham days 4 and 6 < sham day 2 value ($P < 0.05$).

roidogenesis for ganglia extracts from Y-organectomized and sham groups; 65% suppression for ganglia extracts from unoperated controls).

DISCUSSION

We found that injection of 20-hydroxyecdysone increased circulating ecdysteroid titers. The same was found by Adelung (1967) after injecting intermolt crabs with a single dose of ecdysone, followed by extraction of whole animals at intervals and measuring ecdysteroid by the Calliphora assay. He also observed that the ecdysteroid level fell rapidly within the first 4 hours after injection, to 10% of the administered dose, and then, surprisingly, rose again over the subsequent 18 hours to the 50% level before finally declining. These events were interpreted to result first from a rapid clearing of injected ecdysone and then endogenous secretion of ecdysone by the Y-organs, directly stimulated by the injected hormone through a positive feedback mechanism. That the source of the secondary rise in ecdysteroid was endogenous was unequivocally demonstrated: an injection of ^{3}H-ecdysone was cleared rapidly over 4 hours as before but levels of the tracer continued downward over the subsequent time period (Adelung, 1967). In the present study, we show that elevated ecdysteroid levels in crab hemolymph inhibit release of MIH activity from subsequently isolated eyestalk ganglia (Fig. 3). It appears that the better interpretation of Adelung's results, based on present knowledge, is that the secondary rise in ecdysteroids he observed was due to transient inhibition of MIH release induced by the ecdysteroid injection.

A methodology similar to that employed in the present study was used to demonstrate feedback inhibition of ACTH release from rat pituicytes *in vitro* (Mulder and Smelik, 1977); ACTH release from pituicytes of corticosterone-treated rats was measured with a bioassay based on stimulation of steroidogenesis by cultured adrenal cells. In that experiment pituitary cells exhibited the feedback effects for several hours after

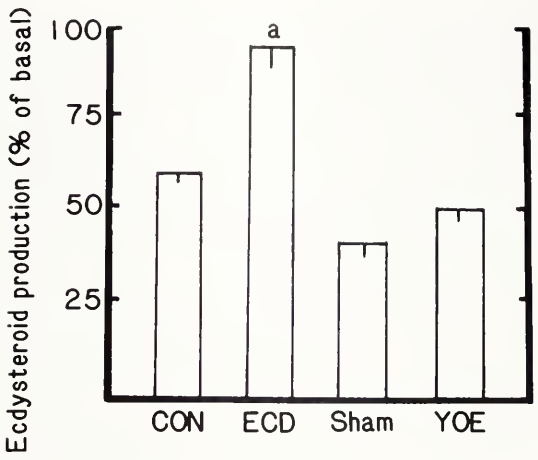

FIGURE 3. Effects of elevated and reduced hemolymph ecdysteroid titers on release of MIH from isolated eyestalk ganglia. Ganglia (removed from the crabs used in the Figs. 1 and 2 experiments) were incubated 2 h in saline, and MIH activity in the conditioned saline was assessed by ability to inhibit Y-organ steroidogenesis. Saline was conditioned with ganglia from untreated control crabs (CON), from crabs with elevated ecdysteroid titers (ECD), from sham-operates (Sham), or from Y-organectomized crabs (YOE). Bars and enclosed lines represent the mean and SEM of 8–10 incubations. Basal ecdysteroid production by Y-organ quarters (in unconditioned saline) averaged 155 ± 12 pg/μg protein/24 h. a, $P < 0.01$–0.001 *vs* CON. Sham *vs* CON, $P < 0.01$.

isolation, indicating a relatively long-lasting influence of corticosteroid exposure *in vivo*. Similarly, eyestalk ganglia isolated from ecdysteroid-treated crabs apparently remained suppressed during 2-h incubations as MIH activity was not detectable in

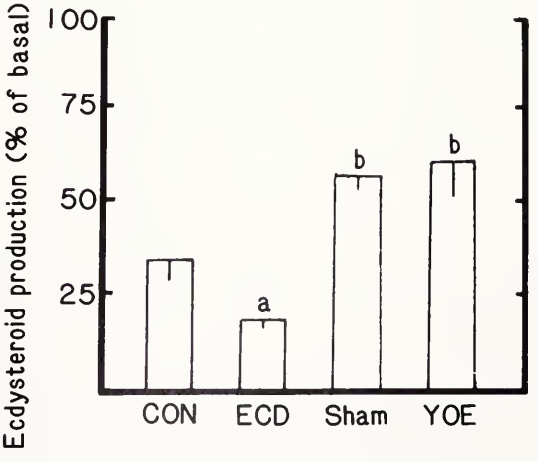

FIGURE 4. Effects of elevated and reduced hemolymph ecdysteroid levels on MIH content of eyestalk ganglia. Y-organ quarters were incubated with extracts of ganglia (2 eyestalk equivalent) from untreated control (CON) crabs, from crabs with elevated ecdysteroid levels (ECD), from sham operates (Sham), or from Y-organectomized crabs (YOE); effects on ecdysteroidogenesis were measured. Bars and enclosed lines represent the mean and SEM of 8–10 incubations. Basal ecdysteroid production by gland quarters averaged 83 ± 12 pg ecdysteroids/μg protein/24 h. All treatments suppressed ecdysteroid production ($P < 0.01$–0.001). a. $P < 0.01$ *vs* CON b. $P < 0.02$–0.01 *vs* CON; $P < 0.001$ *vs.* ECD.

the conditioned saline (Fig. 3). In a previous study we found that as little as 0.01 eyestalk equivalent of MIH activity released from control ganglia in 2-h incubations elicited detectable suppression of Y-organ steroidogenesis; MIH release from feedback-inhibited ganglia was therefore likely less than 1% of levels released from control ganglia. In addition, ganglia from crabs with elevated ecdysteroids contain significantly more MIH activity than ganglia from control intermolt crabs (Fig. 4). The combined results suggest a primary negative feedback effect on MIH release relative to synthesis. Comparable effects were seen in adrenal corticoid feedback on ACTH release; Jones *et al.* (1977) found that corticosteroids inhibited release of ACTH from cultured pituitary cells without affecting its synthesis.

Y-organectomized crabs showed only a 40% reduction in circulating ecdysteroid levels (Fig. 2). Other studies in which Y-organs were ablated and serum ecdysteroid levels were monitored yielded similar results (see Skinner, 1985 for review); one study suggested that ecdysteroids released by ovaries may account for the maintenance of low levels of ecdysteroids in the absence of Y-organs (Lachaise and Hoffman, 1977). While the reduction of ecdysteroid levels caused by Y-organectomy did not affect MIH release (Fig. 3), MIH content of ganglia from Y-organectomized and from sham-operates was reduced (Fig. 4). In light of previous studies indicating that stressors reduce ecdysteroid titers by promoting MIH release (Mattson and Spaziani, 1985c, 1986a) and prevent molting (Aiken, 1969), we propose that stress due to surgery may have caused a large release of MIH prior to post-surgery day 6, resulting in depletion of ganglionic MIH content and low amounts of MIH available for further release.

Serum ecdysteroid levels during crab molting cycles have been determined in several species (Adelung, 1967, 1969; Chang, *et al.*, 1976; Hopkins, 1983; Soumoff and Skinner, 1983; Skinner, 1985) and follow a consistent pattern. Associated with the transition from intermolt to premolt is one or more relatively small, transient rises in hemolymph ecdysteroid titers (3- to 10-fold over intermolt levels). There follows a dramatic rise (100- to 300-fold) that precedes, and presumably initiates, ecdysis. Titers then fall sharply just prior to ecdysis and return shortly thereafter to or below intermolt levels. Results of the present study and of previous work in this and other laboratories suggest interactions of the XO-SG-Y-organ neuroendocrine system that may account for the observed changes in hemolymph titers. The small, pre-ecdysial rises may result from preliminary reduction in MIH secretion due to internal or external environmental cues (*e.g.*, changes in photoperiod and/or temperature, Bliss and Boyer, 1964, Aiken, 1969; intrinsic neuronal oscillators that are species specific, Arechiga *et al.*, 1985; reductions in stressful inputs, Mattson and Spaziani, 1985c, 1986a). Once initiated, the small ecdysteroid peaks may create changes in activity of eyestalk neurosecretory cells or their inputs resulting in feedback inhibition of MIH release (*cf.* Fig. 5). The large secondary rise in ecdysteroids is consequently permitted, causally linked to accentuated feedback inhibition of MIH release (but not synthesis). The magnitude of this rise in ecdysteroids may be explained in part by our recent findings (Mattson and Spaziani, 1986b) that calcium antagonizes MIH suppression of Y-organ activity. It is well documented (*cf.* Greenaway, 1985) that hemolymph calcium titers rise sharply prior to the molt and fall agin, more or less coincident with the changes in ecdysteroids. Thus, both the pre-ecdysial rise and fall in ecdysteroids also may be calcium-linked. In any case, with ecdysteroid levels finally depressed, feedback inhibition on neurosecretory cells would be relieved and MIH release reinstated to dominate the postmolt and intermolt hormonal environments. However, while this scenario may be plausible for crustaceans, it is not apparently consistent with all events in insects, which exhibit the same general pattern of cyclic changes in ecdysteroid levels (*cf.*, Bollenbacher *et al.*, 1975). Steel (1975) provided evidence of negative feedback effects of ecdysteroids

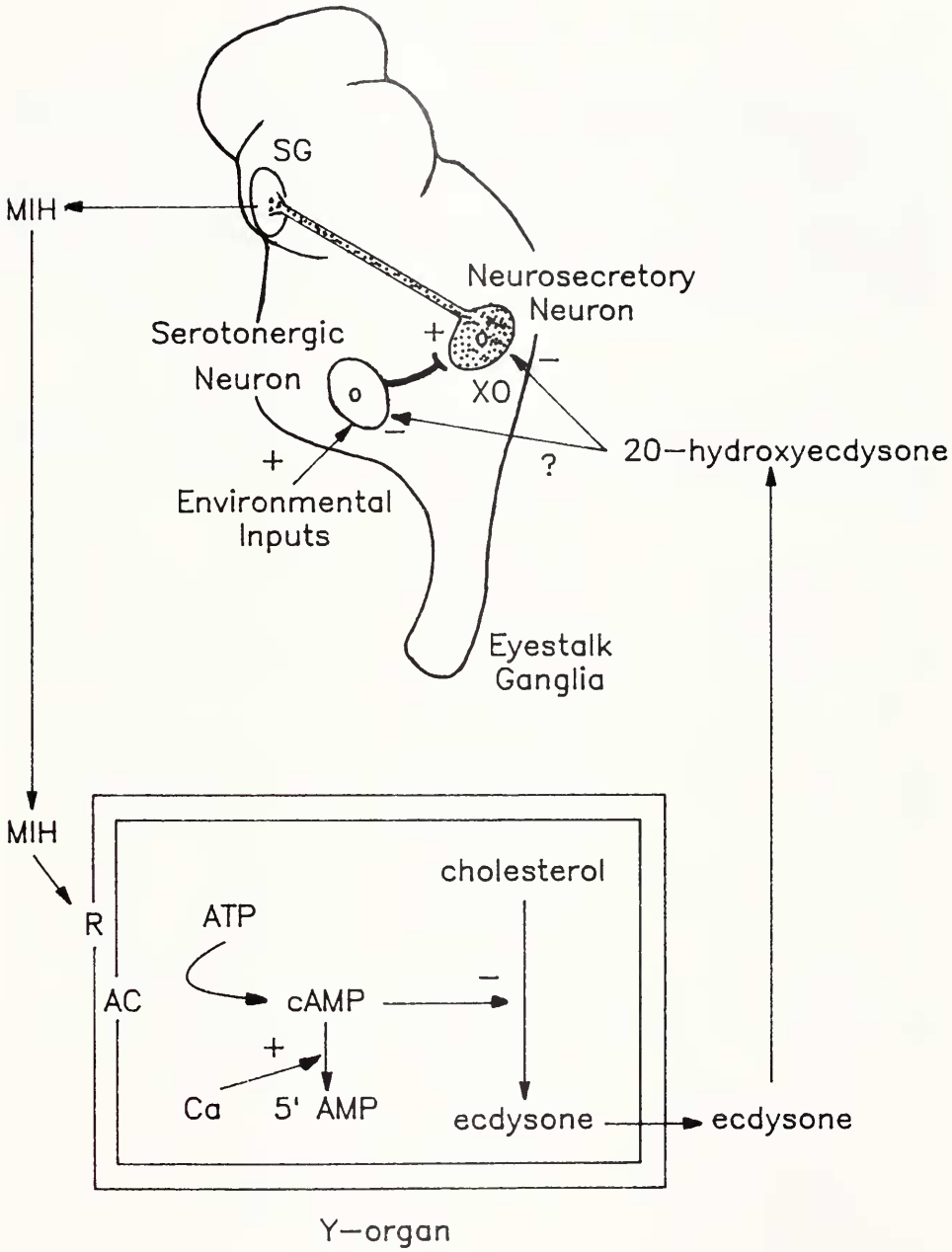

FIGURE 5. Model of neuroendocrine regulatory interactions of the X-organ-sinus gland-Y-organ system. Environmental inputs including stress activate serotonergic eyestalk neurons which stimulate MIH-containing neurosecretory cells of the X-organ (XO)-sinus gland (SG) complex to release MIH. MIH in hemolymph binds to putative Y-organ cell surface receptors (R) resulting in activation of adenylate cyclase (AC) and generation of cAMP. cAMP suppresses production of ecdysone from cholesterol, an effect antagonized by calcium (Ca) which activates a calcium-calmodulin-sensitive cAMP-phosphodiesterase. Thus with continued release of MIH, ecdysone titers remain low and the intermolt state is maintained. A reduction of MIH release (due to a transient increase in hemolymph ecdysteroid levels or to reduced peripheral neural input) releases Y-organs from inhibition (decreased Y-organ cAMP levels) and ecdysone production is increased. Ecdysone is converted in peripheral tissues to 20-hydroxyecdysone. The latter eventually exerts feedback inhibition on release of MIH from XOSG cells, permitting the large rise in hemolymph ecdysteroid titers prior to the molt. Y-organ activity subsequently declines (cause unknown), ecdysteroid titers fall, and MIH is again released reinstating the intermolt stage. See text for further discussion and details.

on release of the insect tropin, PTTH. The problem of consistency in model arises from the fact that the insect tropin stimulates ecdysteroid secretion whereas the crustacean tropin is inhibitory. Thus, the initial rises in ecdysteroids in insects, and the subsequent large increase, would be expected to result from a stimulation, not suppression, of PTTH release. In further contrast with crustaceans, the subsequent pre-ecdysial fall in ecdysteroids is more satisfactorily explained in insects by the feedback inhibition hypothesis. Clearly, resolution of these questions must await the development of a sensitive means for measuring arthropod tropic hormone levels in hemolymph as a function of the molting cycle.

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